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The Bactericidal And Cytotoxic Effects Of Antimicrobial Wound Cleansers

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Objective: Wound care is a part of daily activity for many athletic trainers. Knowing which cleansers are effective against the bacteria that most commonly cause infection and whether they are toxic to healthy cells enables athletic trainers to make educated decisions on which cleanser to use. We compared the bactericidal effectiveness and cytotoxicity to human fibroblast cells of 4 cleansers at various dilutions.

Design and Setting: A 4 × 4 factorial design was used for the cytotoxicity testing. The independent variables were type and dilution of cleanser. The dependent variable was cell viability of the human fibroblast cells. We used a 2 × 3 × 4 × 4 factorial design for the bacterial testing. The independent variables were type and dilution of bacteria and type and dilution of cleanser. The dependent variable was the bactericidal action of the cleanser on the bacteria.

Subjects: Human foreskin samples were used to obtain a line of fibroblast cells. Bacterial samples were obtained from an athletic training clinic, isolated from swabs of a whirlpool water supply valve (*Pseudomonas aeruginosa*) or skin surface (*Staphylococcus aureus*).

Measurements: We obtained bactericidal measurements by testing isolated Gram-negative (*Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) bacteria. Minimum and maximum concentrations were identified according to bactericidal effectiveness. Cytotoxicity measurements were obtained from spectrophotometer readings of a neutral red assay for fibroblast cell viability. Final dilutions tested were determined by pilot testing.

Results: At the 1:5 dilution of product in sterile 0.9% saline, both Cinder Suds and Nitrotan and hydrogen peroxide were different from the control with regard to *Pseudomonas aeruginosa.* At the 1:10 dilution, both Betadine and hydrogen peroxide were different from the control with regard to *Pseudomonas aeruginosa.* These 2 cleansers were also different from each other. At the 1:10 dilution, only Betadine was not different from the control for the cytotoxicity testing.

Conclusions: Betadine was both effective against bacteria and not harmful to human fibroblast cells at a 1:10 dilution of a commercially purchased solution.

Key Words: wound cleansers, cytotoxicity, bactericidal, antimicrobial

As health care workers, athletic trainers encounter a variety of different kinds, shapes, and sizes of wounds that need to be cleaned or debrided (or both) on a daily basis.[1–5] Knowing how to treat these wounds is crucial for several reasons. First, providing an optimal healing environment results in decreased rates of infection and faster healing.[1–5] Second, the appropriate method and materials to cleanse a wound are somewhat controversial.[1–7] There are 2 areas of concern when considering which cleanser to use. The first area is the efficacy of the cleanser against the bacterial flora that may be encountered in a wound.[1–8] Using a cleanser that is effective against bacteria can aid the healing process.[1–3,8–11] However, the second area of concern is that the cleanser not inhibit the cells that are involved in the healing process.[1–3,8–11] Health care professionals do not always consider the effects of wound cleansers on the healthy tissue. Recent studies[1–3,8–11] have shown that many cleansers currently used in the athletic training field are cytotoxic to the cells necessary to the healing process. Ideally, health care professionals want a cleanser that is effective against bacteria but has no inhibitory effects on the healing tissues. Therefore, our purpose was to evaluate 4 cleansers (Cinder Suds and Nitrotan [Cramer Products, Inc, Gardner, KS], hydrogen peroxide, Betadine [The Purdue Frederick Co, Norwalk, CT], and saline) and compare their effectiveness against bacteria (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) and their toxicity to healthy human cells (fibroblasts).

METHODS

We used a 2 × 3 × 4 × 4 factorial design for the bactericidal assay. The independent variables were the type of bacteria (*P aeruginosa* or *S aureus*), the concentration of bacteria (1×10^7, 1×10^8, and 2.5×10^8), the type of cleanser

(Betadine, hydrogen peroxide, Cinder Suds and Nitrotan, and 0.9% saline as the control), and the dilution (1:5, 1:10, 1:50, and 1:100 product in saline). The dependent variable was bacterial toxicity.

We used a 4 × 4 factorial design for the cytotoxicity assay. The independent variables were the type of cleanser and the dilutions of the cleansers tested (1:5, 1:10, and 1:50).

Subjects

Human fibroblast cells were obtained from the Indiana University School of Medicine Department of Dermatology. The primary cell line of fibroblasts was established as an explant from human foreskin samples. Once the flasks were confluent with a monolayer of cells (the bottom of the flask fully covered), the cells were harvested and some were frozen for later use. The fibroblast cells were used for all human cytotoxicity determinations. Exemption from the Human Subjects Committee was obtained because primary human cell cultures were used for all testing measures.

Instruments

We used a spectrophotometer (Model #U-2000, Hitachi Ltd, Tokyo, Japan) to measure cell viability via neutral red bioassay. The spectrophotometer measures light absorbency at set wavelengths. An inverted phase-contrast microscope (Model ELWD 0.3, Nikon Corp, Tokyo, Japan) was used to detect cell confluency and growth in tissue culture flasks. A light microscope (Model ATC 2000, Nikon Corp, Buffalo, NY) was used for cell counting. A 37° incubator supplied with 5% CO_2 (Model 023, Forma Scientific, St. Louis, MO) provided optimal growing conditions for fibroblast cells. A laminar flow hood (LabGard Model NU427, NuAire Inc, Plymouth, MN) was used to ensure sterile working conditions. A centrifuge (Model GPR, Beckman Instruments Inc, Palo Alto, CA) was used for collecting cells from various cultures by centrifugation.

Testing Procedures

The Cinder Suds and Nitrotan wound cleansers were donated by the manufacturer; we purchased the other cleansers commercially. The manufacturer's suggestion is to use Cinder Suds to assist with cleansing of the wound and then to saturate a sterile gauze pad and lay it on the wound for several minutes. This allows the ingredients to seep into the skin and the wound. All cleansers were diluted in cell culture medium for the fibroblast testing and in saline for all bacterial testing.

Tissue Culture

Tissue culture methods and neutral red bioassay were modeled after Cooper et al.[12] Human fibroblasts were isolated from human foreskin samples. The tissue sample was minced in a sterile 0.15 M saline solution containing 1% trypsin for enzymatic separation of epidermis from dermis. To isolate individual human fibroblasts, 0.025% trypsin + 0.01% ethylenediaminetetraacetic acid (EDTA) was used. The human fibroblasts were grown in complete Dulbeccos' Modified Eagle's Medium ([DMEM] Gibco BRL, Bethesda, MD) containing 10% fetal bovine serum (FBS) 10 ng/mL epidermal growth factor (Imcera Inc, Terre Haute, IN), and penicillin (10 000 U/mL)-streptomycin (10 000 mg/mL) as an antibiotic. To ensure that the pH of the cleanser did not interfere with cell viability, pH measurements were obtained for each agent as well as for the control (media with no cleanser added).[13,14]

Cytotoxicity Determination Procedure

The neutral red assay was chosen because previous testing has demonstrated it to be a sensitive measure of cell viability.[15–17] Subconfluent human fibroblasts (2×10^3 cells/mL) were grown in secondary cultures in complete DMEM + 10% FBS (without antibacterial agents). The human fibroblasts were harvested and placed into 96-well, flat-bottom tissue culture plates and incubated for 3 days to establish cell confluency. Antibacterial agents were diluted in DMEM + 10% FBS (growth medium) and placed into the wells. The diluted cleansers were allowed to remain in contact with the cells for 15 minutes, and then the medium was removed. Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) was added to each well, and the plates were placed in the incubator for 3 hours. The cells were then washed with formol-calcium (10 mL 40% formaldehyde, 10 mL 10% anhydrous calcium chloride, and 80 mL water) and fixed using an acetic acid-ethanol mixture (1.0 mL glacial acetic acid in 100 mL 50% ethanol), the dye extracted using a repeating pipetter, and the absorbency at 540 nm measured (A_{540}) using a spectrophotometer. The uptake of neutral red is proportional to the number of viable (live) cells.[18] The control was medium without an added test agent.

Bactericidal Procedures

Serial dilutions of each agent were made in filtered sterile saline. Two log concentrations were tested at half-log intervals. Concentrations of 1:5, 1:10, 1:50, and 1:100 were tested.

The method used by Lineaweaver et al[14] was modified for this experiment. Samples of bacteria were obtained from one of the whirlpools in an athletic training room. Pure cultures were evaluated by Gram staining with microscopic evaluation and biochemical species identification. Paired bacterial suspensions (each containing 1×10^7, 1×10^8, or 2.5×10^8 organisms) of *P aeruginosa* and *S aureus* were cultured separately in nutrient broth. Bacteria were suspended in 3.0 mL of either a topical agent or saline for 15 minutes. The suspensions with either topical agent or saline were then centrifuged at 2000 × g for 5 minutes and resuspended in 3.0 mL of saline. The suspensions were plated on nutrient agar culture medium (Difco Labs, Bethesda, MD) and incubated for 24 hours at 37°C. Colonies present after the 24-hour culture were counted. Colony counts were coded (0 = no growth, 1 = 1 to 30 colonies, 2 = 31 to 100 colonies, 3 = 101 to 200 colonies, 4 = 201 to 300 colonies, and 5 = >300 colonies), and the coded data was used for statistical analysis. Concentrations as specified for each test agent were used to determine maximal and minimal bactericidal concentrations.

STATISTICAL ANALYSIS

We used an analysis of variance to test for statistical significance of cytotoxicity. We also used univariate F tests and the Tukey procedure for post hoc testing. Results for the bactericidal study were coded and analyzed using the Kruskal-Wallis test. Post hoc testing was done using the Mann-Whitney U test. A probability level of $P \leq .05$ was set for all tests.

Table 1. Results of Bactericidal Testing

Agent	Control	Dilution			
		1:5	1:10	1:50	1:100
Control	5.0 ± 0.0				
Betadine		0.0 ± 0.0*	0.0 ± 0.0*	1.8 ± 1.3*	3.7 ± 2.5
Hydrogen peroxide		2.6 ± 2.1	3.2 ± 2.5	5.0 ± 0.0	5.0 ± 0.0
Cinder Suds and Nitrotan		2.2 ± 2.5*	3.6 ± 1.6	5.0 ± 0.0	5.0 ± 0.0

*Significantly different from control bactericidal results ($P \leq .05$).

Table 2. Cytotoxicity Results

Agent	pH	Control	Dilution		
			1:5	1:10	1:50
Control	7.5	0.2776 ± 0.0062			
Betadine	7.7		−0.0066 ± 0.0028*	0.1396 ± 0.068	0.4281 ± 0.0068
Hydrogen peroxide	7.6		−0.0068 ± 0.0045*	−0.0030 ± 0.0054*	0.4071 ± 0.0079
Cinder Suds and Nitrotan	7.7		−0.0004 ± 0.1034*	−0.0027 ± 0.0059*	0.1233 ± 0.0062*

*Significantly different from control cytotoxicity results ($P \leq .05$).

RESULTS

Bactericidal Results

We found no differences among the bacterial concentrations tested (Table 1). Differences were found between bacterial growth and dilution of cleansers ($X^2_4 = 14.9$, $P = .005$) and between bacterial growth and type of cleanser ($X^2_3 = 13.0$, $P = .005$). No differences were found for *S aureus* at any of the dilutions and for *P aeruginosa* at dilutions 1:50 and 1:100. Statistically significant differences were revealed for *P aeruginosa* between the dilutions of 1:5 ($X^2_3 = 7.8$, $P = .05$) and 1:10 ($X^2_3 = 7.6$, $P = .05$).

Post hoc testing using the Mann-Whitney U test for nonparametric data revealed no differences at the dilution 1:5 between Betadine and Cinder Suds and Nitrotan, Betadine and hydrogen peroxide, Betadine and control, and Cinder Suds and Nitrotan and hydrogen peroxide. Statistically significant differences at the 1:5 dilution were found between Cinder Suds and Nitrotan and control ($U < .001$, $P = .03$) and hydrogen peroxide and control ($U < .001$, $P = .03$). No differences were found at the 1:10 dilution between Betadine and Cinder Suds and Nitrotan, Cinder Suds and Nitrotan and hydrogen peroxide, and Cinder Suds and Nitrotan and control. There were statistically significant differences at the 1:10 dilution between Betadine and hydrogen peroxide ($U < .001$, $P = .03$), Betadine and control ($U < .001$, $P = .03$), and hydrogen peroxide and control ($U < .001$, $P = .03$).

Cytotoxicity Results

Cytotoxicity testing showed an interaction between type and dilution of cleansers (Table 2; $F_{4,23} = 7.4$, $P = .001$). Simple main-effects testing revealed that at a 1:5 dilution, Betadine ($F_{3,9} = 18.5$, $P = .01$), hydrogen peroxide ($F_{3,9} = 18.5$, $P = .002$), and Cinder Suds and Nitrotan ($F_{3,9} = 18.5$, $P < .001$) were all different from the control. At the 1:10 dilution, hydrogen peroxide ($F_{3,9} = 13.2$, $P = .002$), and Cinder Suds and Nitrotan ($F_{3,9} = 13.2$, $P = .002$, SE = .047) were both different from the control. Betadine was not different from the control at the 1:10 dilution. At the 1:50 dilution, there were still significant differences between the Cinder Suds and Nitrotan and the control.

DISCUSSION

Previous research[1–5,7–11,19–23] has shown that if an antimicrobial wound cleanser does not kill bacteria, the risk of infection is significantly higher. We used a Gram-negative (*P aeruginosa*) and a Gram-positive (*S aureus*) bacterium for bactericidal testing procedures. The bacterial samples were obtained from an athletic training room whirlpool and skin surfaces. Pure cultures were verified by Gram staining, and the bacteria were identified using biochemical testing. We chose these 2 bacteria for several reasons. First, together they represent a large spectrum of the bacteria that cause skin wound infections. *S aureus* is present as a normal flora organism on the skin surfaces of most healthy humans.[19]*P aeruginosa* is a ubiquitous environmental organism associated with whirlpool folliculitis, an infection caused by immersion in contaminated water.[20,24] Athletic trainers may encounter wound infections caused by either of these common bacteria that are representative of the 2 main bacterial categories. In addition, the cell walls of the 2 bacteria are very different. Each has a peptidoglycan protective layer, but in *P aeruginosa*, this layer is only 10 nm thick, whereas in *S aureus*, it is 80 nm thick.[20] The thicker this peptidoglycan layer, the more resilient the bacteria are to surface-active antimicrobial agents.[19,20] Because we found no significant differences among any of the cleansers for the *S aureus* bacteria, we can hypothesize that the cleansers may not contain strong enough ingredients to be effective against Gram-positive bacteria. Both the hydrogen peroxide and the Cinder Suds and Nitrotan were more specifically bactericidal for the *P aeruginosa*; that is, they were more effective at killing that bacterial type versus the *S aureus*. Betadine was equally effective against both bacteria. Although not statistically significant, there were numeric differences in Betadine's effectiveness against the 2 bacteria versus the other cleansers.

We chose the cleansers to be tested based on our experience in the athletic training environment and from the results of the Goldenberg[1] study. Goldenberg showed that many athletic

trainers are currently using Betadine and hydrogen peroxide for wound cleansing.[1] Cinder Suds and Nitrotan were chosen because they are marketed specifically to athletic trainers in various catalogues. The chosen cleansers have a variety of different active ingredients. Betadine contains 10% povidone iodine, which is equivalent to 1% available iodine in the marketed, undiluted product. Hydrogen peroxide solution contains 3% stabilized hydrogen peroxide. Cinder Suds contains water, soap, isobutene, and propane. Nitrotan contains picric acid (0.2% vol/vol), tannic acid (1.5% vol/vol), benzyl alcohol (6.1% wt/vol), and isopropyl alcohol (62% wt/vol). Finally, Betadine was the only cleanser that was similar to the control at the 1:10 dilution for cytotoxicity testing. It was also bactericidal at this concentration. This is the ideal combination for an antimicrobial wound cleanser. It kills the bacteria that cause infection, but even more importantly, it is not toxic to the fibroblast cells critical to the healing process. This could be a result of the active ingredient; none of the other cleansers tested have povidone iodine as an active ingredient.

Betadine was safe at a diluted concentration. At a 1:5 dilution, it was toxic to the human fibroblast cells in this study, and by extrapolation, the commercially purchased concentration would be toxic to human fibroblast cells as well. Thus, it is important to note that in order for Betadine to be nontoxic for human fibroblast cells, it must be diluted. For a 1:10 dilution, 1 part Betadine and 9 parts sterile saline should be mixed. This dilution is beneficial in many ways. Not only does it create an optimal healing environment by killing bacteria while sparing fibroblast cells, but a single purchase also lasts much longer. This lowers costs, as it is somewhat expensive to use concentrated Betadine and few athletic training budgets are unlimited.

The literature is currently divided as to the effectiveness of these antimicrobial cleansers. Some researchers[2,4] suggest that Betadine at certain concentrations is very effective and causes minimal damage to healthy tissue. However, others[4,6,7,14,15,21] believe that Betadine's toxicity against the healthy tissue is greater than its bactericidal effectiveness. Hydrogen peroxide's effectiveness is under discussion as well. Several investigators[2,14,22,23,25] have demonstrated that hydrogen peroxide's bactericidal effectiveness is minimal, while its cytotoxicity is very high. Others[3,26] have suggested that at higher dilutions, it may not be as toxic to the healthy tissues but most likely is still ineffective against bacteria. Our study is the first to address bactericidal effectiveness and cytotoxicity of Cinder Suds and Nitrotan, although Nitrotan is advertised to ''prevent infection and promote healing.''

Clinical Relevance

As health care professionals, athletic trainers need to be aware not only of the bactericidal effects of an antimicrobial wound cleanser but also of the cytotoxic effects on healthy human cells. Our results demonstrate that Betadine in saline at a 1:10 dilution of the commercially purchased solution was effective in killing both Gram-positive and Gram-negative bacteria, yet it was not harmful to normal fibroblast cells. As such, we recommend this 1:10 dilution be used in practice to provide the optimal balance between the bactericidal and cytotoxic effects. Further, this dilution is an excellent cost-cutting measure.

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